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(54) Title: METHOD FOR IDENTIFYING COMPOUNDS THAT INDUCE AN INCREASED LEVEL OF THE NERVE GROWTH FACTOR mRNA

(57) Abstract

The invention features an *in vitro* method for identifying a compound capable of increasing the level of NGF in a mammalian cell. The method involves (a) introducing a plasmid DNA containing the NGF promoter into a cell, wherein the promoter is functionally linked to a reporter gene; (b) exposing the cells to the compound; and (c) measuring the level of reporter gene product produced by exposure of the cells to the compound, an increase in the level of reporter gene product indicating an increase in the activity of the NGF promoter, and thereby indicating that the compound is capable of increasing the level of NGF in the mammal. The method is useful for the treatment of diseases associated with atrophy of cholinergic neurons, e.g., Alzheimer's Disease or for the treatment of sensory peripheral neuropathies.

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# METHOD FOR IDENTIFYING COMPOUNDS THAT INDUCE AN INCREASED LEVEL OF THE NERVE GROWTH FACTOR mRNA

## Background of the Invention

This invention relates to an *in vitro* method of identifying a compound that induces an increased level of the Nerve Growth Factor (NGF) messenger RNA (mRNA).

Alzheimer's disease is an intractable and progressively incapacitating dementia that affects the lives of as many as four million patients in the United States alone. The neuropathology of Alzheimer's disease is marked by the degeneration of basal forebrain cholinergic neurons and their associated axons (Whitehouse et al., *Science* 215:1237-1239 (1982)). This neuronal loss is correlated with a decrease in the level of cortical acetyltransferase, which is, in turn, believed to be responsible for the loss of cognitive function characteristic of the disease (Perry et al., *Br. Med. J.* 2:1457-1459 (1978)). To date, therapeutic strategies that utilize drugs in an attempt to elevate cholinergic function have been unsuccessful (Coyle et al. *Science* 219:1184-1190 (1983)).

NGF is a neurotrophic agent for basal forebrain cholinergic neurons (Whittemore et al. *Brain Res. Rev.* 12:439-464 (1987)) leading to the suggestion that neurotrophic factor therapy might retard or prevent the loss of basal forebrain cholinergic neurons. Cholinergic neurons express the NGF receptor, enabling them to respond to the beneficial effects of NGF (Hefti et al., *Neurosci Lett.* 69:37-41 (1986)). The results of animal model studies demonstrate that administration of exogenous NGF can prevent the death of cholinergic neurons after axotomy (Hefti, *J. Neurosci* 6:2155-2162 (1986); Williams et al., *Proc. Natl. Acad. Sci. USA* 83:9231-9235 (1986)) and can enhance the function of remaining cholinergic neurons in

aging animals (Fischer et al., *Nature* 329:65-68 (1987)).

One considerable difficulty with using NGF as a potential therapeutic for Alzheimer's disease is that the protein does not cross the blood-brain barrier and thus must be administered directly into the cerebrospinal fluid by chronically implanted catheters. Catheter administration does not lend itself to treatment of a large number of patients. Thus it becomes desirable to administer compounds which could cross the blood-brain barrier and enhance the cellular production of NGF. Support for the feasibility of this approach comes from studies demonstrating that small organic compounds such as 1,25 - dihydroxyvitamin $D_3$, TPA (12-O-tetradecanoylphorbol - 13 - acetate), and catecholamine derivatives stimulate the production of NGF in fibroblasts (Wion et al., *J. Neurosci. Res.* 28:110-114 (1991); Wion et al., *FEBS Lett.* 262:42-44 (1990); Furukawa et al., *FEBS Lett.* 247:463-467 (1989)). These compounds increase levels of NGF mRNA.

The promoter of the mouse NGF gene contains sequences characteristic of eukaryotic gene regulatory elements (Zheng et al., *Mol. Brain Res.* 3:133 -140 (1988)). These include consensus CAAT and TATA box elements, known to be essential for basal level gene transcription, as well as an AP-1 element which acts as a binding site for the transcription factor composed of the Fos and Jun proteins. Factors that stimulate the production of the Fos and Jun proteins also elevate the expression of some genes whose promoters contain AP-1 elements (Curran et al., *Cell* 55:395-397 (1988)). The AP-1 site, present in the first intron of the NGF gene, mediates the induction of NGF transcription in response to treatment with the phorbol ester TPA (D'Mello et al., *Mol. Cell. Neurosci.* 2:157-167 (1991)).

## SUMMARY OF THE INVENTION

The invention features a method for identifying a compound that increases the level of NGF protein in a mammal, e.g., a human. The method involves (a) introducing a recombinant DNA molecule that includes the NGF promoter into a cell, wherein the promoter is functionally linked to a reporter gene; (b) exposing the cells to the compound; and (c) measuring the level of reporter gene product produced by exposure of the cells to the compound, an increase in the level of reporter gene product indicating an increase in the amount, i.e., synthesis and/or stability, of the reporter gene mRNA, and thereby indicating that the compound is capable of increasing the level of NGF in the mammal. As a preferred embodiment, the promoter further includes a deletion of a transcriptional suppressor element.

An "increase in the level of NGF" is indicated in this assay by an increase in the level of reporter gene product, which in turn indicates an increase in levels of reporter gene mRNA due to sequences in the NGF promoter. The "increase in the level of reporter gene product" is shown by comparing the measured amount of reporter gene product in cells exposed to the test compound relative to the level of reporter gene product measured in cells that are not exposed to the test compound. These cells experience the same conditions (minus the test compound) as the cells that are exposed to the test compound, and thereby serve as a negative control. The increased level of reporter gene product is generally 1-10 fold, preferably 10-100 fold, that of the level of reporter gene product of the negative control.

The sensitivity of the method of the invention to compounds that have a positive inductive effect on NGF transcription has been optimized by deleting a region(s) of nucleic acid sequence from the promoter that otherwise

would have a negative effect on the level of promoter activity. This region(s) is referred to herein as "a transcriptional suppressor element". The deleted region is preferably a portion of the upstream nucleic acid sequence that serves as a binding site for a compound, e.g., a protein, that, when bound, induces this negative effect. (The negative effects of the transcription suppressor elements(s) are further discussed herein, and are illustrated in Figure 3.) Applicants have shown that by using DNA constructs that include an NGF promoter lacking the suppressor element(s), the response of the assay to the test compound is thereby increased.

The method of the invention can also be used to identify compounds that elevate reporter gene expression by blocking the function of the transcriptional suppressor elements. Promoter constructs containing the targeted negative elements can be used to identify active compounds.

The deletion of the transcriptional suppressor element(s) can comprise any region of the upstream sequences shown to have a negative regulatory effect on the promoter, for instance those provided as examples by applicants. Deleted regions are also those sequences specifically identified as suppressor binding sites by methods known to those skilled in the art and by those methods provided herein. Deleted regions are also those sequences whose removal results in enhanced NGF promoter activity, e.g., nucleotides -63 to -735 of the upstream sequence of the NGF gene, or a portion thereof. Preferred portions can include, in addition to the deleted regions provided herein, deletions of 5-1000, 10-150, or 20-50 nucleotides, or a combination of deletions of any size that results in enhanced NGF promoter activity. By "upstream sequence of the NGF gene" is meant any region of the first 6000 nucleotides that are adjacent to the naturally occurring human NGF gene in the 5′ direction.

An "NGF promoter", as used herein, is a nucleic acid sequence, e.g., 50-1000 nucleotides in length, that is necessary for initiation of transcription of the adjacent protein coding sequences. Because the NGF promoter region may also include transcribed sequences, these sequences may also enhance NGF mRNA stability. By a "recombinant DNA molecule", as used herein, is meant a DNA vector, e.g., a plasmid, a virus, or an insertional element, e.g., a transposon, that has been altered to include the NGF promoter and reporter gene as described herein, and that includes sequences enabling it to be introduced into, and stably maintained by, a host cell. By "compound" is meant an inorganic molecule, an organic chemical compound, a protein, or a peptide fragment of, e.g., 5-10, 10-30, or 30-90, amino acids.

In other preferred embodiments the reporter gene is a gene that produces a protein product that is easily measured in a routine assay. Suitable reporter genes known to those skilled in the art include chloramphenicol acetyltransferase (CAT), luciferase, and beta-galactosidase. The CAT gene is preferred. Convenient assays are, e.g., an enzymatic activity assay, a colorimetric assay, or a fluorometric assay; examples of routine assays used to measure the concentration of reporter gene product are provided in the examples below. Most preferred are reporter genes that are expressed within the cell and whose products are measured in the intracellular medium, or in an extract of the intracellular medium of a cultured mammalian cell line. This provides advantages over using a reporter gene product that is secreted from the cell and measured in the conditioned medium, since the rate and efficiency of secretion introduce an added variable and thereby complicate interpretation of the assay.

The invention also features a method of improving

the function of cholinergic neurons in a mammal, e.g., a human. The method involves administering to the mammal a compound that increases the activity of the NGF promoter in a neuronal tissue of the mammal, e.g., the brain, spinal cord, nerve or anatomically related cells, i.e., cells in close proximity to a part of a neuron. Such cells include muscle cells, smooth and skeletal; epithelial cells such as keratinocytes; Schwann cells; fibroblasts; astrocytes; glandular cells, e.g., submandibular, adrenal, prostate, thyroid, and testis; iris; heart; vas deferens; and spleen cells. Fetal cell implants are known to induce NGF formation. Preferably, the compound is identified by the NGF promoter transcription assay described herein. "Cholinergic neurons" are defined as cells that employ acetylcholine as their neurotransmitter. "A method of improving the function of cholinergic neurons" refers to a method that results in an increased level of acetylcholine produced and/or released from cholinergic neurons. As a preferred embodiment, the mammal bears a cellular transplant. The cellular transplant preferably contains neurons or adrenal cells.

The invention also features a method of treating, e.g., Alzheimer's disease, or any other disease that exhibits dysfunction or atrophy in cholinergic neurons in a mammal, e.g., a human. The method involves administering to the mammal a compound that increases the level of NGF protein in a neuronal tissue of the mammal, e.g., the brain, spinal cord, nerve or anatomically related cells. Preferably, the compound is identified by the NGF promoter transcription assay described herein. As a preferred embodiment, the mammal bears a cellular transplant. The cellular transplant preferably contains neurons or adrenal cells.

The invention also features a method of treating Parkinson's disease in a mammal bearing a cellular

transplant. The method involves administering to the mammal a compound that increases the level of NGF protein in or around the transplant of the mammal. The cellular transplant may contain neurons or adrenal cells. Preferably, the compound is identified by the NGF promoter transcription assay described herein.

The invention also features a method of treating a peripheral neuropathy of sensory nerves, whether caused by a disease, e.g., diabetes, or as a consequence of tissue injury or administration of a toxic agent, e.g., a cancer chemotherapeutic agent such as cisplatin or taxol. In addition, the invention features a method for enhancing the survival of transplanted, e.g., a cellular transplant of, neurons or adrenal cells, e.g., following their transplantation into the brains or cerebral ventricles of patients with Parkinson's disease or Alzheimer's disease. The various methods involve administering to the mammal a compound that increases the level of NGF protein in or near the brain, spinal cord, or nerve or other peripheral tissue of the mammal. Preferably, the compound is identified by the NGF promoter transcription assay described herein.

Also provided within the scope of the invention is a recombinant DNA fragment, molecule, or plasmid that includes an NGF promoter, wherein the promoter is functionally linked to a reporter gene, the reporter gene bereft of its native promoter. In preferred embodiments, the promoter includes a deletion of a transcriptional suppressor element. The deletion includes any portion of the upstream sequence of the NGF gene, i.e., any of the regions listed above, that is shown to contain sequences that have a negative effect on the transcriptional activity of the NGF promoter. The reporter gene is any gene that produces a protein product that can be measured in a routine assay as described above, examples including, but not limited to, the chloramphenicol acetyltransferase (CAT)

gene, the luciferase gene, and the beta-galactosidase gene.

Also included is a cell, e.g., a cultured eukaryotic cell, or a prokaryotic cell, i.e., a bacterial cell, that includes the recombinant DNA molecule used in the NGF promoter transcriptional assay of the invention. The cell can be suitable for use in this assay, or can simply be used as a storage host for the recombinant DNA molecule. Where the host is a prokaryotic cell, the DNA molecule contains sequences for the stable maintenance and replication of the DNA molecule in the cell, as described by the art (Ausubel et al. eds. Current Protocols in Molecular Biology, John Wiley & Sons, publ. NY. 1987, 1989, hereby incorporated by reference; Sambrook et al. (1989), Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, hereby incorporated by reference). The DNA molecule is preferably stably integrated into the chromosomal DNA of the cell, but would also be functional for the purposes stated herein in the form of an extrachromosomal DNA element.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

## Detailed Description

The drawings are first described.

### The drawings

Fig. 1 is a schematic illustration of the construction of the novel CAT reporter vector pCBE-36.

Fig. 2 is an illustration of the restriction sites used to generate the NGF promoter/CAT reporter plasmids shown in the lower portion of the figure. Nucleotides are numbered, with +1 representing the site of transcription initiation. SA designates the splice acceptor site and CAT represents the chloramphenicol acetyltransferase coding sequences.

Fig. 3 is a graphical illustration of the CAT

enzymatic activity measured in cell extracts of cells hosting the NGF promoter/CAT reporter plasmids. The values shown are the averages of three separate transfections with the standard deviations designated by error bars. CAT enzymatic activity is expressed as "% acetylation."

Fig. 4 is a graphical illustration comparing levels of CAT enzymatic activity displayed by the NGF promoter-CAT reporter gene constructs p121-CAT and pCB-121 (expressed as "% acetylation"). The values shown are the averages of two separate transfections with the ranges designated by error bars.

Fig. 5 is a graphical illustration of the NGF promoter/CAT reporter plasmid p735-CAT after TPA-mediated induction. The values shown are the averages of two separate transfections with the ranges designated by error bars.

The Invention

Applicants have recognized the need for compounds that can cross the blood brain barrier to increase NGF production *in situ*, and have therefore developed a rapid and efficient screening assay to facilitate the search for such compounds. In order to do so, applicants engineered a novel DNA plasmid comprising an NGF promoter functionally linked to a reporter gene, the plasmid also including sequences for the proper expression of the reporter gene in a eukaryotic cell line. Applicants foresaw the necessity of optimizing the assay by further defining the structure of the NGF promoter, identifying regions of the promoter that are inducible, and identifying regions of the promoter that have a negative effect on transcription. A summary of the procedure applicants followed to design the assay is presented, followed by a detailed description of the steps taken to engineer the plasmid and to test its effectiveness for screening compounds that induce transcription from the NGF promoter.

In order to define the structure of the NGF promoter and investigate the inducible nature of the promoter sequences, the mouse NGF promoter region was isolated and cloned into a novel reporter plasmid. The plasmid contains a bacterial CAT gene which is dependent upon adjacent NGF promoter sequences for transcription initiation. Unlike conventional CAT reporter plasmids, this modified CAT vector contains an RNA splice acceptor site, derived from the SV40 virus genome, that mates with the RNA splice donor site provided by the cloned NGF promoter DNA fragment. Inclusion of the acceptor site facilitates the correct splicing of the CAT mRNA and permits the inclusion of the NGF AP-1 site in the promoter-reporter constructions. If applicants had not included the acceptor site, potential aberrant splicing of the CAT mRNA caused by the presence of a splice donor site and the absence of a corresponding acceptor site would likely have invalidated the results of the promoter studies presented herein (Huang et al., *Mol. Cell. Bio.* 10:1805-1810 (1990)).

Applicants further modified the CAT reporter plasmid by replacing the SV40 early polyadenylation signal with the SV40 late polyadenylation signal. This exchange has been shown to increase steady-state levels of mRNA in another system by increasing the efficiency of 3′ end formation (Carswell et al., *Mol. Cell. Biol.* 9:4248-4258 (1989)). These modifications result in an overall increase in sensitivity of the present assay.

After modifying the CAT reporter plasmid the NGF promoter fragment was inserted into it, and the resulting constructions was transfected into mouse L929 cells (A.T.C.C. #CCL1). Applicants have tested the effectiveness and sensitivity of the assay by conducting a positive control. Promoter activity is measured in transient expression assays by quantitating the levels of CAT enzymatic activity present in cellular extracts. Cells

containing the NGF promoter-reporter plasmid were treated with the phorbol ester TPA, resulting in an increase in CAT activity. Increased CAT activity was not seen in similarly treated cells containing a promoterless CAT plasmid. Therefore TPA induced reporter-specific mRNA levels in this assay by an NGF promoter-specific mechanism.

Applicants have thereby demonstrated the utility of this system for the identification of compounds that induce NGF mRNA levels by acting on NGF promoter sequences. This method can provide compounds useful as novel therapeutics for the treatment of pathologies that exhibit cholinergic neuronal dysfunction or atrophy. Such pathologies include, for example, Alzheimer's disease.

The method can further provide compounds useful as novel therapeutics for treating sensory peripheral neuropathies as well as for promoting the survival of NGF-sensitive transplanted neurons or adrenal cells.

Example 1. GENOMIC CLONING AND DNA SEQUENCING

A mouse liver genomic library (Promega, Madison, WI) is screened with a 60 bp oligonucleotide probe homologous to the 5' end of the mouse NGF gene corresponding to bases 1-60 of the mouse NGF mRNA (Selby et al., *Mol. Cell. Bio.* 7:3057-3064 (1987)). A lambda phage clone that hybridizes with the probe was purified, providing a 7.0 kb BamHI restriction fragment which was then cloned into the BamHI site of the plasmid pGEM-11Zf+ (Promega, Madison, WI), generating the plasmid designated pBU2. The sequence composition of pBU2 is confirmed by partial sequencing using the chain termination reaction method (Sanger et al., *Proc. Natl. Acad. Sci. USA* 74:5463-5467 (1977)) and Sequenase enzyme (US Biochemicals, Cleveland, OH).

Example 2. CONSTRUCTION OF THE NGF-CAT GENE REPORTER PLASMID

Referring to Fig. 1, the CAT reporter plasmid pCAT-BASIC (Promega) was digested with the enzyme EcoRI to produce 2.97 and 1.5 kb fragments. The 1.5 kb fragment was replaced with a 700 bp EcoRI fragment from the plasmid pL-LPA (Carswell et al. 1989 supra) to form the construct pCBE-1. This fragment exchange replaced the SV40 virus large T antigen splice signals and the SV40 early polyadenylation signal with the SV40 late polyadenylation signal. pCBE-1 was digested with the restriction enzyme XbaI and the 5′ overhangs were blunt-ended with the Klenow fragment of DNA polymerase. A 41 bp HincII/NcoI restriction fragment containing the 19S splice acceptor signal from the SV40 viral genome was also blunt-ended by Klenow enzyme treatment and ligated to the XbaI linear pCBE-1 vector. The resulting plasmid construct is designated pCBE-36. pCBE-36 was sequenced by the chain termination reaction method to confirm the orientation of the inserted DNA fragments and sequence composition.

Example 3. CONSTRUCTION OF THE NGF-CAT GENE REPORTER PLASMIDS WITH VARIABLE DELETIONS IN THE NGF PROMOTER

The pCBE-36 plasmid DNA was digested with the restriction enzyme SalI. The 5′ overhangs were blunt-ended with Klenow enzyme treatment. Six DNA restriction fragments derived from the NGF promoter region present in the plasmid pBU2 were isolated and blunt-ended by Klenow treatment. These fragments include: a) an 855 bp SphI/XhoI fragment; b) a 597 bp EcoRV/XhoI fragment; c) a 445 bp BanI/XhoI fragment; d) a 372 bp EcoRI/XhoI fragment; e) a 241 bp HaeIII/XhoI fragment; and f) a 183 bp EcoRI/XhoI fragment. Each DNA fragment was ligated to a SalI linear pCBE-36 plasmid DNA molecule in six separate reactions to yield six NGF promoter deletion-CAT reporter plasmids. The

promoter region of each of the six plasmids is illustrated in Fig. 2. The clones were characterized by restriction enzyme mapping to confirm the orientation of the inserted DNA fragments. Each plasmid DNA was purified by column chromatography (Qiagen, Chatsworth, CA).

Example 4. DEMONSTRATION OF FUNCTIONAL PROMOTER ACTIVITY IN THE NGF PROMOTER-CAT GENE REPORTER PLASMIDS

To determine the promoter activity of the plasmid constructions shown in Fig. 2, the plasmid DNA molecules were introduced into mouse L929 fibroblast cells. L929 cells (5 x $10^5$ cells per 100 mm dish) were seeded 48 hrs prior to transfection. Cells were washed with serum-free MEM-alpha medium (Gibco, Gaithersburg, MD) immediately prior to Lipofectin (Gibco/BRL)-mediated transfection (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417 (1987)). Plasmid DNA (2.5 μg) was combined with 55.0 μg Lipofectin reagent in a final volume of 110 μl and added to cells in 7.0 ml serum-free MEM-alpha medium. Cells were incubated for 16 hrs at 37° C in an atmosphere of 5% $CO_2$. Sixteen hours after transfection, 3.3 ml of MEM-alpha medium containing 30% horse serum was added to each culture dish and the cells were incubated an additional 24 hours. Cells were harvested 48 hours after transfection by scraping the cells into a solution of 2.5 ml phosphate-buffered saline. The cells were pelleted by centrifugation, resuspended in 50 μl 250 mM Tris-HCl pH 7.5, and lysed by three rounds of freezing and thawing. Cellular debris was removed from the extract by centrifugation. Protein content in the supernatant was determined by the Bradford assay (Bio-Rad, Hercules, CA). Aliquots of cell extract (100 μg per reaction) were assayed for CAT enzymatic activity by the method of Gorman et al. (*Mol. Cell. Biol.* 2:1044-1051 (1982), hereby incorporated by reference). Each of the six NGF promoter constructions,

as well as the promoterless vector pCBE-36, was analyzed for promoter activity in this fashion. Fig. 3 shows that all six of the NGF promoter-CAT reporter plasmids were active in producing some CAT enzymatic activity. However, there was a progressive decrease in promoter activity with longer 5' upstream sequences beyond nucleotide -121. The promoterless plasmid did not produce significant levels of CAT activity. These data indicate that the cloned NGF promoter sequences contain functional regulatory elements that contribute to the transcription of the adjacent CAT gene. This indicates the presence of negative regulatory elements within upstream sequences.

Example 5. DEMONSTRATION OF INCREASED ASSAY SENSITIVITY ASSOCIATED WITH USE OF THE MODIFIED CAT REPORTER PLASMID pCBE-36

A comparative study was conducted to determine how the modifications performed on pCAT-BASIC to generate pCBE-36 influence CAT assay sensitivity. In particular, applicants wished to know whether inclusion of a splice acceptor site on the plasmid improved the efficiency of the assay. The 241 bp HaeIII/XhoI NGF promoter fragment was subcloned into pCAT-BASIC to form pCB-121. Hence, both pCB-121 and p121-CAT contained the NGF promoter region up to nucleotide -121, but pCB-121 lacks the splice acceptor site. Extracts derived from cells transfected with pCB-121 or p121-CAT differed in their levels of CAT enzymatic activity (Fig. 4). Cells transfected with p121-CAT displayed approximately 15-fold higher levels of acetylation activity than did those transfected with pCB-121. This finding indicates that the modifications introduced into pCAT-BASIC to generate pCBE-36 lead to a significant increase in CAT assay sensitivity. The enhanced sensitivity is useful in the study of weak promoter elements previously uncharacterized due to low assay sensitivity associated with the use of conventional

CAT reporter vectors.

Example 6. INDUCTION OF NGF PROMOTER ACTIVITY WITH THE PHORBOL ESTER TPA

As a positive test for whether the NGF promoter sequences contain regulatory elements that can induce steady-state mRNA above basal levels, the transient expression assays were repeated using the promoter construct p121-CAT and the promoterless plasmid pCBE-36. Following transfection, cells were treated with serum containing a final concentration of 100 ng/ml TPA. Control reactions were treated with serum alone. As shown in Fig. 5, CAT activity in cells transfected with p121-CAT increased approximately two-fold in the presence of TPA compared to untreated controls. Cells transfected with the promoterless construct were unaffected by TPA treatment. The results indicate that the isolated NGF promoter sequences contain a TPA-responsive element. This element most likely correlates with the intronic AP-1 binding site (D'Mello et al., supra).

Example 7. ASSAYS FOR MEASURING THE LEVEL OF REPORTER GENE PRODUCT

The method of measuring the level of reporter gene product is appropriate to the reporter gene product used on the recombinant DNA molecule in the NGF promoter assay. For example, where the reporter gene encodes an enzyme, an enzymatic assay is used. The concentration of reporter gene product is determined by providing excess substrate for that enzyme, under conditions in which the enzyme concentration is the limiting factor on the reaction, and measuring the concentration of product produced. In one example, the CAT gene (Promega, Madison, WI) encodes the enzyme chloramphenicol acetyltransferase, the level of which can be determined by providing acetyl CoA and chloramphenicol and measuring the production of acetylated

chloramphenicol. When luciferase is used as the reporter gene (Promega, Madison, WI), the cells hosting the recombinant DNA molecule are lysed, adenosine triphosphate (ATP) and luciferin are added to the lysate in a liminometer, and the resulting light emission is correlated with the amount of luciferase present in the lysate (Nordeen, *BioTechniques* 6:454-457 (1988)). Beta-galactosidase (An et al., *Mol. Cell. Bio.* 2:1628-1632 (1982)) (Promega, Madison, WI) is measured with chromogenic substrates, i.e., colorless substrates which are hydrolyzed to yield colored products, e.g., o-nitrophenyl-β-D-galactoside (Promega, Madison, WI), which are then measured spectrophotometrically or by the formation of colored bacterial colonies. More detailed descriptions of reporter gene assays are available in the art (Ausubel et al. eds. Current Protocols in Molecular Biology, John Wiley & Sons, publ. NY. 1987, 1989, pp. 9.6.1-9.6.14, hereby incorporated by reference; Miller et al. Experiments in Molecular Genetics Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1972. pp. 352-355, hereby incorporated by reference). Other reporter genes and assays for their corresponding protein products are equally suitable and known to those skilled in the art.

Example 8. METHOD OF IDENTIFYING NEGATIVE SUPPRESSOR SEQUENCES IN THE NGF PROMOTER

Negative suppressor sequences can be defined using standard deletion analyses combined with reporter gene assays. For example, a restriction fragment extending approximately 1.0 kb upstream from the NGF coding sequences is inserted into the CAT reporter vector pCBE-36 (construct p735-CAT in Fig. 2) and assayed for reporter gene activity. A series of related NGF promoter-reporter gene fusion plasmids are constructed, each containing successive 5′ deletions of NGF sequences (see Fig. 2), and assayed in a similar fashion. Potential negative suppressor sequences

will confer reduced promoter activity to reporter constructs containing these sequences (e.g., construct p735-CAT), while reporter plasmids lacking these sequences (e.g., p252-CAT, p121-CAT) will exhibit enhanced activity (see Fig. 2 and Fig. 3 for example). Negative suppressor sequences identified by deletion analysis (bases -735 to -122) can be isolated by cleavage with restriction enzymes (SphI and HaeIII) and inserted into promoter-reporter gene plasmids which exhibit known strong activity (p121-CAT for example). The resulting constructs display reduced activity due to the dominant negative effect of suppressor elements present in the inserted sequences.

The existence of negative suppressor elements in the NGF promoter can be further demonstrated using *in vivo* competition assays (Schöler et al., *Cell* 36:403-411 (1984); Loh et al., *J. Virol.* 62:4086-4095 (1988)). The restriction fragment spanning the putative negative suppressor regions (-735 to -122) is cloned into the promoterless plasmid pGEM 5Zf+ (Promega, Madison, WI) to generate construct pNEG. Cells are transfected with the NGF promoter-reporter fusion construct p735-CAT and co-transfected with increasing amounts of pNEG DNA. The low level of activity exhibited by p735-CAT alone gradually increases as the level of co-transfected pNEG DNA increases. This effect is due to the titration of DNA-binding proteins responsible for the negative transcriptional effect away from the promoter-reporter gene plasmid DNA and binding to the negative suppressor sequences in the pNEG DNA. This competition for negative DNA-binding factors further verifies the presence of suppressor elements in the NGF promoter.

Example 9. METHOD OF IDENTIFYING COMPOUNDS THAT INCREASE THE LEVEL OF NGF IN A HUMAN PATIENT

The method of the invention is used as an *in vitro* screening procedure to identify compounds that increase NGF

mRNA levels, and thereby identify compounds that would increase the level of NGF in a mammal, e.g., in a human patient. The compounds positively identified in the screening method can be re-tested in an additional *in vivo* assay designed to confirm that increased NGF mRNA levels by the compound results in higher NGF levels in a mammal. Examples of appropriate *in vivo* assays include a method of measuring endogenous NGF mRNA levels in rat brains in response to chemical induction (Fabrazzo et al., *Mol. Pharm.* 39:144-149 (1991)), and a method of measuring endogenous NGF protein levels in tissue using anti-NGF antibodies (Korsching et al., *Proc. Natl. Acad. Sci. USA* 80:3513-3516 (1983)).

Example 10. METHOD OF EVALUATING THE FUNCTION OF CHOLINERGIC NEURONS IN A HUMAN PATIENT

The ability of a compound of the invention to enhance cholinergic neuron function is assessed via behavioral and/or electrophysiological testing methods which are well known to those versed in the art. For forebrain cholinergic neuronal function, which is impaired in Alzheimer's disease, there is a well established correlation between extent of memory loss and extent of acetylcholine decline. Thus memory tests, such as those described by Perry et al., supra, can be used to determine the extent to which a compound of the invention enhances basal forebrain cholinergic neuronal function. For spinal cord motor neuron cholinergic function, which is impaired in amyotrophic lateral sclerosis, there is a well established correlation between extent of motor function disability and extent of acetylcholine decline. Thus, motor tests, such as described by Appel et al., *Annals of Neurology* 22:328-333 (1987), can be used to determine the extent to which a compound of the invention enhances spinal cord cholinergic motor neuronal function. Measurement of the level of acetylcholine released is described by Fonnum

et al., *J. Neurochem.* 24:407-409 (1975).

EXAMPLE 11.: METHOD OF EVALUATING THE FUNCTION OF SENSORY PERIPHERAL NERVES IN A HUMAN PATIENT

The ability of a compound of the invention to reduce peripheral neuropathy in sensory nerves is carried out by administering said compound to said patient in an effective dosing schedule and then measuring sensory nerve activity according to methods which are well known to those versed in the art, e.g., a combination of electrophysiological and neurological examination testing methods such as are described in DeAngelis et al., *Cancer* 67:2241-2246 (1991).

Other embodiments are within the following claims.

What is claimed is:

CLAIMS

1. A method for identifying a compound that increases the level of NGF protein in a mammal, said method comprising

(a) introducing a recombinant DNA molecule comprising the NGF promoter into a cell, wherein said promoter is functionally linked to a reporter gene;

(b) exposing said cells to said compound; and

(c) measuring the level of reporter gene product produced by exposure of said cells to said compound, an increase in said level of reporter gene product indicating an increase in the amount of the reporter gene mRNA, and thereby indicating that said compound is capable of increasing the level of NGF in said mammal.

2. The method of claim 1, wherein said promoter further comprises a deletion of a transcriptional suppressor element.

3. The method of claim 1, wherein said reporter gene product is measured in the intracellular medium of said cell.

4. The method of claim 1, wherein said reporter gene is the chloramphenicol acetyltransferase (CAT) gene.

5. The method of claim 1, wherein said reporter gene is the luciferase gene.

6. The method of claim 1, wherein said reporter gene is the beta-galactosidase gene.

7. The method of claim 1, wherein said mammal is a human.

8. Use of a compound that increases the activity of the NGF promoter in a neuronal cell in the preparation of a medicament for improving the function of cholinergic neurons.

9. The use of claim 8, wherein said mammal bears a cellular transplant.

10. The use of claim 9, wherein said cellular transplant contains neurons.

11. The use of claim 9, wherein said cellular transplant contains adrenal cells.

12. The use of claim 8, wherein said mammal is a human.

13. Use of a compound that increases the level of NGF protein in a neuronal tissue, or in an anatomically related cell, in the preparation of a medicament for treating Alzheimer's disease.

14. The use of claim 13, wherein said mammal bears a cellular transplant.

15. The method of claim 14, wherein said cellular transplant contains neurons.

16. The method of claim 14, wherein said cellular transplant contains adrenal cells.

17. Use of a compound that increases the level of NGF protein in or around a transplant of a mammal in the preparation of a medicament for treating Parkinson's disease in a mammal bearing a cellular transplant.

18. The use of claim 17, wherein said cellular transplant contains neurons.

19. The method of claim 17, wherein said cellular transplant contains adrenal cells.

20. Use of a compound that increases the level of NGF protein in or near nerve tissue in the preparation of a medicament for treating peripheral neuropathy.

21. The use of claim 20, wherein said mammal bears a cellular transplant.

22. The use of claim 20, wherein said cellular transplant contains neurons.

23. The use of claim 22, wherein said cellular transplant contains adrenal cells.

24. A recombinant DNA fragment comprising an NGF promoter, wherein said promoter is functionally linked to a reporter gene, said reporter gene bereft of its native promoter.

25. The DNA molecule of claim 24, wherein said promoter comprises a deletion of a transcriptional suppressor element.

26. The DNA molecule of claim 24, wherein said reporter gene is the chloramphenicol acetyltransferase (CAT) gene.

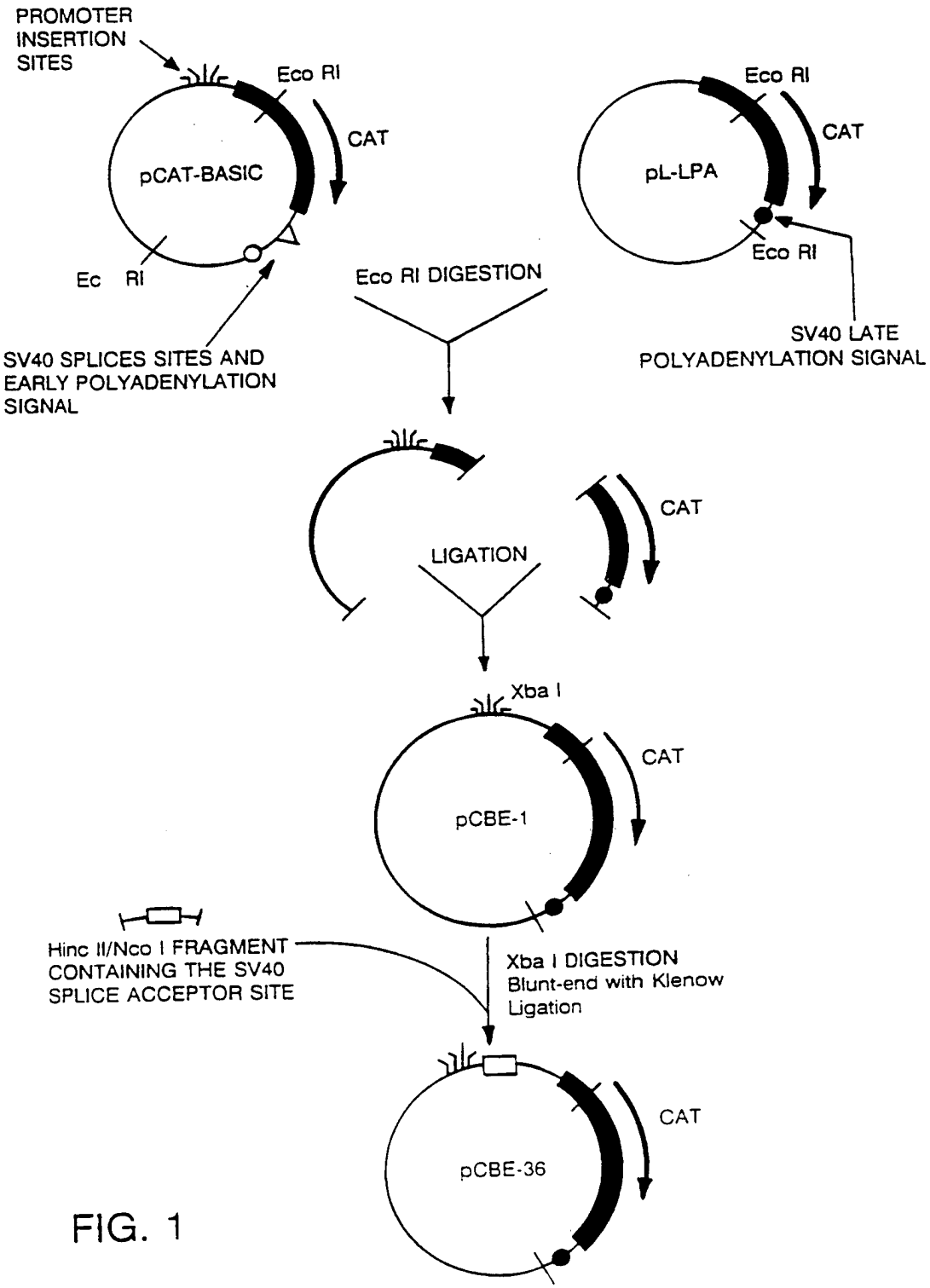
27. The DNA molecule of claim 24, wherein said reporter gene is the luciferase gene.

28. The DNA molecule of claim 24, wherein said reporter gene is the beta-galactosidase gene.

29. A cell comprising the recombinant DNA molecule of claim 24.

30. The cell of claim 29, wherein said cell is a cultured eukaryotic cell.

31. The cell of claim 29, wherein said recombinant DNA molecule is integrated into the chromosomal DNA of said cell.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CAT
pCAT-BASIC
Ec RI
SV40 SPLICES SITES AND EARLY POLYADENYLATION SIGNAL
Eco RI DIGESTION
Eco RI
CAT
pL-LPA
Eco RI
SV40 LATE POLYADENYLATION SIGNAL
CAT
LIGATION
Xba I
CAT
pCBE-1
Hinc II/Nco I FRAGMENT CONTAINING THE SV40 SPLICE ACCEPTOR SITE
Xba I DIGESTION
Blunt-end with Klenow
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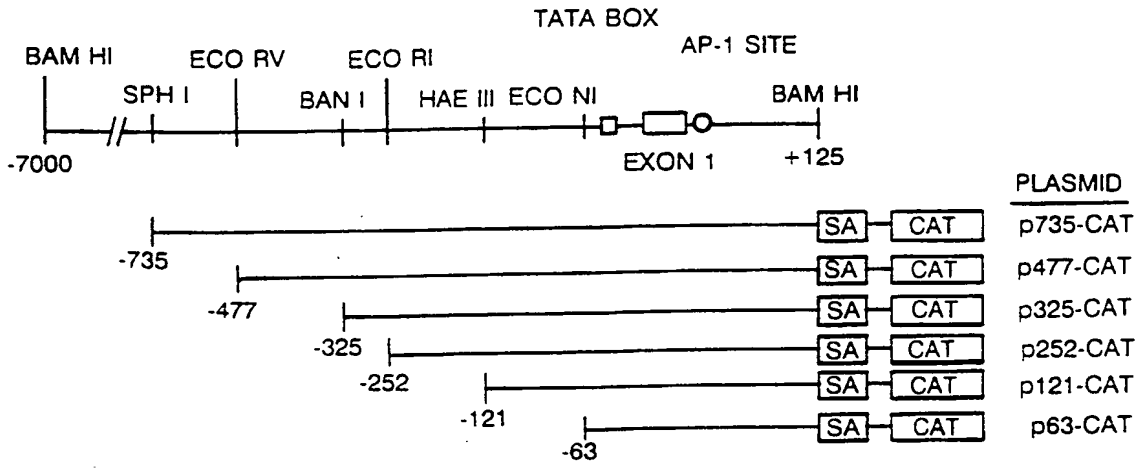
FIG. 1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BAM HI
ECO RV
ECO RI
SPH I
BAN I
HAE III
ECO NI
BAM HI
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EXON 1
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PLASMID
SA
CAT
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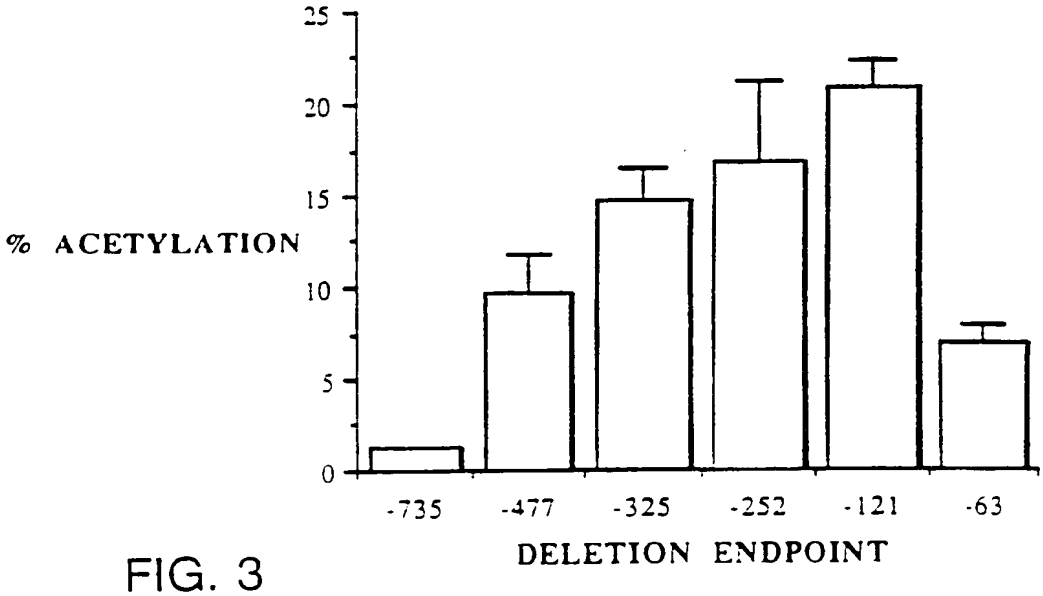
p63-CAT

FIG. 2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-63
DELETION ENDPOINT

FIG. 3

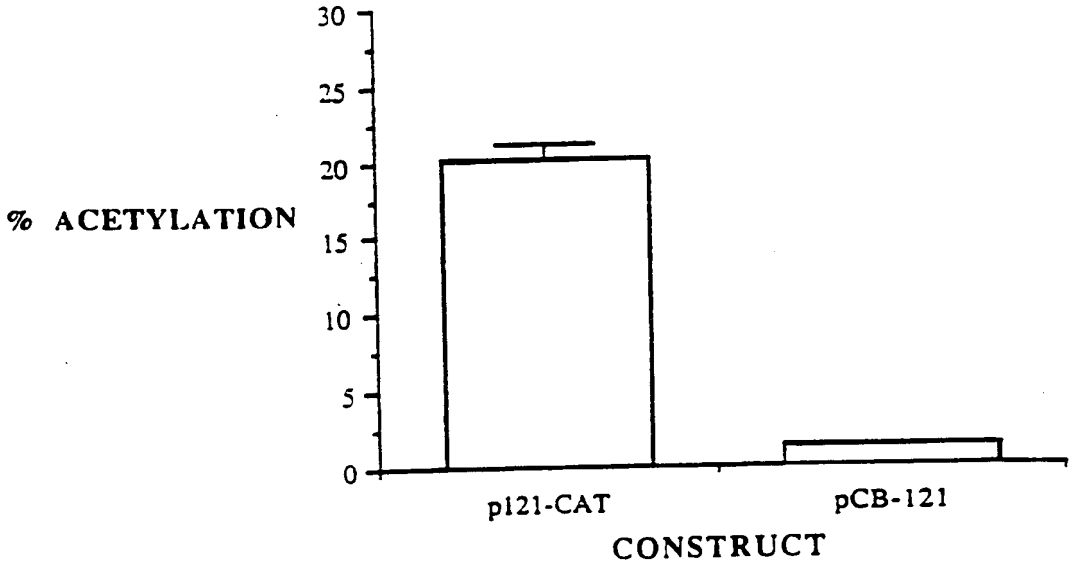


FIG. 4

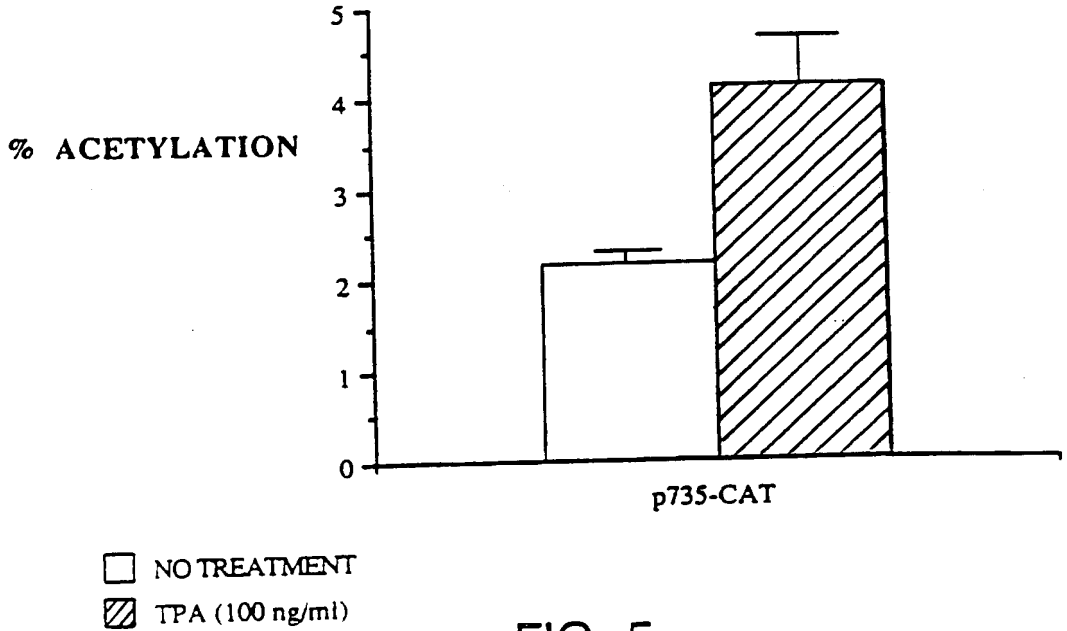


FIG. 5

# INTERNATIONAL SEARCH REPORT

International application No. US94/00818

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :C12N 15/11, 15/85; C12P 21/00, 21/02; C12Q 1/02, 1/66

US CL :435/6, 8, 29, 69.1, 70.1; 536/23.2, 23.7, 24.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 8, 29, 69.1, 70.1, 172.1, 172.3; 536/23.2, 23.7, 24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Molecular Brain Research, Volume 15, issued 1992, Carswell et al., "Induction of NGF by isoproterenol, 4-methylcatechol and serum occurs by three distinct mechanisms", pages 145-150, see especially page 146. | 1-31 |
| Y | Molecular and Cellular Biology, Volume 2, number 12, issued December 1982, An et al., "Expression of Bacterial ß-Galactosidase in Animal Cells", pages 1628-1632, see entire article. | 6, 28 |
| Y | BioTechniques, Volume 6, issued 1988, S. K. Nordeen, "Luciferase Reporter Gene Vectors for Analysis of Promoters and Enhancers", pages 454-457, see entire article. | 1-31 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be part of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>02 March 1994 | Date of mailing of the international search report<br>25 MAR 1994 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. NOT APPLICABLE | Authorized officer<br>ROBERT HODGES Jill Warden for<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNA IAL SEARCH REPORT

International application No.
PCT/US94/00818

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Molecular and Cellular Biology, Volume 2, number 9, issued September 1982, Gorman et al., "Recombinant Genomes Which Express Chloramphenicol Acetyltransferase in Mammalian Cells", pages 1044-1051, see entire article. | 4, 26 |
| Y | Molecular and Cellular Biology, Volume 7, number 9, issued September 1987, Selby et al., "Mouse Nerve Growth Factor Gene: Structure and Expression", pages 3057-3064, see especially Fig. 3. | 1-31 |
| Y | Molecular Brain Research, Volume 3, issued 1988, Zheng et al., "Structural and functional analysis of the promoter region of the nerve growth factor gene", pages 133-140, see especially Fig. 1. | 1-31 |
| Y | FEBS Letters, Volume 247, number 2, issued April 1989, Furukawa et al., "Catecholamines increase nerve growth factor mRNA content in both mouse astroglial cells and fibroblast cells", pages 463-467, see especially last paragraph of page 466. | 1-31 |
| Y | Moelcular Brain Research, Volume 11, issued 1991, D'Mello et al., "Structural and functional identification of regulatory regions and cis elements surrounding the nerve growth factor gene promoter", pages 255-264, see especially Fig. 1. | 2, 25 |
| Y | FEBS Letters, Volume 262, number 1, issued March 1990, Wion et al., "Phorbol 12-myristate 13-acetate (PMA) increases the expression of the nerve growth factor (NGF) gene in mouse L-929 fibroblasts", pages 42-44, see entire article. | 1-31 |
| Y | Journal of Neuroscience Research, Volume 28, issued 1991, Wion et al., "1,25-Dihydroxyvitamin D-3 Is a Potent Inducer of Nerve Growth Factor Synthesis", pages 110-114, see entire article. | 1-31 |